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Options of partners improve carbon for phosphorus trade in the arbuscular mycorrhizal mutualism

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Abstract

The mutualism between plants and arbuscular mycorrhizal fungi (AMF) is widespread and has persisted for over 400 million years. Although this mutualism depends on fair resource exchange between plants and fungi, inequality exists in partners despite preferential allocation favoring beneficial partners and sanctions inhibiting cheaters. Here we use ^{33}P and ^{14}C isotopes and a split-root system to test for preferential allocation and reciprocal rewards in the plant–AMF symbiosis by presenting a plant with two AMF that differ in cooperativeness. We found that plants received more ^{33}P from less cooperative AMF in the presence of another AMF species. This increase in ^{33}P resulted in a reduced ^{14}C cost per unit of ^{33}P from less cooperative AMF when alternative options were available. Our results indicate that AMF diversity promotes cooperation between plants and AMF, which may be an important mechanism maintaining the evolutionary persistence of and diversity within the plant–AMF mutualism.

Key words

plant–AMF mutualism, microbial diversity, split-root system, isotopes, biological markets, supply and demand

Introduction

Biological market models are frequently used to understand resource exchange and the ecological persistence of cooperation among mutualists (Noë & Hammerstein 1994, 1995; Schwartz & Hoeksema 1998; Kummel & Salant 2006; Kiers & Denison 2008; de Mazancourt & Schwartz 2010). Although these models provide numerous predictions regarding the mechanisms that promote the ecological persistence of mutualisms, few empirical tests of resource exchange in mutualisms exist (Denison 2000; Kiers *et al.* 2011; Grman 2012; Verbruggen *et al.* 2012; Walder *et al.* 2012). Therefore, direct evidence of the underlying mechanisms that maintain mutualisms is scarce, especially for mutualisms involving a high diversity of species that span a range of cooperative behaviour such as the plant–arbuscular mycorhizal fungi (AMF) mutualism (Sachs *et al.* 2004; Kiers & Van Der Heijden 2006; Walder & van der Heijden 2015).

Numerous theories have been applied to cooperation within mutualisms that are mainly developed from biological market, game and resource-ratio theory (Noë & Hammerstein 1994; Hoeksema & Bruna 2000; Foster & Wenseleers 2006; de Mazancourt & Schwartz 2010; Archetti *et al.* 2011). A few predictions have developed from these theories that are important for the maintenance of mutualisms: 1) cooperator association whereby cooperative genotypes of the two partners preferentially associate with each other allowing cooperative genotypes to persist, 2) partner-fidelity feedbacks whereby benefits from a partner enhance the other that then pass those benefits on to the first partner and 3) partner choice whereby the partners involved in the mutualism actively control the association (Sachs *et al.* 2004; Foster & Kokko 2006; Foster & Wenseleers 2006; Archetti *et al.* 2011). Overall, mutualisms will persist if the costs are low relative to the benefits (Foster & Wenseleers 2006; de Mazancourt & Schwartz 2010). However, resource availability and supply and demand of resources from other potential mutualists will alter the cooperation between partners (Noë &

68 Hammerstein 1994; Grman 2012). Furthermore, these feedbacks may not be mutually
69 exclusive and may operate simultaneously at different temporal and spatial scales.

70 Partner choice is an important component maintaining the plant–AMF mutualism
71 (Kiers & Van Der Heijden 2006; Kiers & Denison 2008; Kiers *et al.* 2011; Walder & van der
72 Heijden 2015). It has been suggested that both plants and AMF can discriminate between
73 different mutualist partners and in turn preferentially allocate resources to more beneficial
74 partners (Helgason *et al.* 2002; Fitter 2006; Kiers & Van Der Heijden 2006; Bever *et al.* 2009;
75 Kiers *et al.* 2011). Sanctions and rewards in mutualisms are thought to be involved as
76 enforcement mechanisms that stabilize mutualistic interactions (Kiers & Van Der Heijden
77 2006). For example, Denison (2000) found sanctions by legumes against less-effective
78 rhizobial partners. Similarly, both Bever *et al.* (2009) and Kiers *et al.* (2011) separately
79 showed that plants preferentially allocate carbon to more cooperative AMF partners.
80 Therefore, when infected by multiple AMF, vascular plants can select more effective partners
81 (Yoneyama *et al.* 2007; Kiers & Denison 2008). In addition, exchange of luxury goods,
82 functional diversity and sink strength have also been suggested to explain resource exchange
83 in the plant–AMF symbiosis (Walder & van der Heijden 2015).

84 Although partner choice can maintain the plant–AMF mutualism, it is not clear how
85 plants and AMF recognize exploiters (Bronstein 2001; Archetti *et al.* 2011). The process of
86 partner selection is further complicated by variation in environmental conditions, differences
87 in specific plant–AMF combinations and the type of resource being exchanged (Grman 2012;
88 Grman *et al.* 2012; Walder & van der Heijden 2015). Therefore, how sanctions and rewards
89 mediate plant response to multiple AMF species, even in the exchange of a single resource or
90 with only a few AMF species, remains unclear.

91 In our study, we used a simplified novel microcosm design that integrated a split-root
92 system (Coutts & Philipson 1976) and a partitioned rhizosphere (e.g. a root compartment, an

93 AMF compartment and an outer nutrient-labelling compartment; Fig 1) to assess carbon
94 allocation and phosphorus acquisition by a single plant in response to one or two AMF
95 species. Dual ^{14}C and ^{33}P radio-labelling allowed us to track resource exchange in the plant–
96 AMF mutualism. By using two plant species that differ in their mycorrhizal responsiveness in
97 combination with two AMF species that vary in their beneficial effects on these two plant
98 species (Wagg *et al.* 2011), we could test the effect of variable supply and demand economies
99 on the plant–AMF mutualism. We hypothesized that carbon and phosphorus exchange
100 depended on the identity of the AMF exchanging resources with the plant and the AMF
101 competing for resource exchange on the opposite side of the root. In this concept, plants
102 mediate resource exchange by interpreting the supply and demand of the AMF species present
103 on the root system and responding with sanctions or rewards.

104 **Materials and Methods**

105 *Design of split-root systems*

106 We grew host plants in microcosms and separated their root systems into two halves
107 by means of a polyvinyl chloride (PVC) wall (Fig. 1). Each side of the split-root system was
108 inoculated with one of two AMF species or was an un-inoculated control. Both sides of the
109 root-system were further partitioned into three 200 mL compartments at increasing distance
110 from the plant center (0–2 cm, 2–4 cm, 4–6 cm) by 20-µm nylon mesh. This mesh allowed
111 hyphae but not roots to pass through. Roots could therefore only colonize the two central
112 compartments while AMF could colonize all three compartments of a side. The outermost
113 compartment was used to supply phosphorus that could only be accessed by the plant via the
114 AMF. This setup allowed us to examine carbon investment from the plant to the AMF in
115 exchange for the phosphorus acquired by the plant from the AMF partner.

116 *Plant and AMF species*

Soil from a natural grass-clover field (pH 6.7, Agroscope Reckenholz research station, Zürich, 47° 25'N, 8°31'E) was sieved through a 5 mm mesh and mixed with quartz-sand (1:9 v/v). The mixture was sterilized by gamma irradiation (25–80 kGy, LEONI, Aargau, Switzerland) and filled into the central four microcosm compartments. The outermost compartments were filled with a polystyrene spacer to reduce desiccation and prevent algal growth. Immediately prior to the application of the phosphorus tracer (^{33}P), the polystyrene was removed and filled with the same soil mixture.

Seeds of *Plantago lanceolata* L. (ribwort plantain; a less responsive mycorrhizal plant) and *Trifolium pratense* L. (red clover; a more responsive mycorrhizal plant) (FENACO, Switzerland) were surface-sterilized by soaking in 5% aqueous hypochlorite solution for 10 min before rinsing four times with demineralized water and germination in sterile sand. The emerging seedlings were transplanted to individual pots and grown for four weeks until they were transplanted to the split-root microcosms. During transplanting the main roots were clipped 2 cm below the shoot to promote lateral root growth. In each microcosm, two plants of the same species were supported with a 3 cm long quartz-sand filled PVC tube that rested on the central PVC sheet dividing the root system. We used two individuals per microcosm to average differences in individual performance and symmetry and to ensure sufficient plant material for all the destructive measurements required to track resource-exchange (see below).

Each of the two middle compartments of each side were amended with *Funneliformis mosseae* (Krüger *et al.* 2012), *Rhizophagus irregularis* (Krüger *et al.* 2012) or remained AMF-free with all three pairwise combinations realized (see Wagg *et al.* 2011 for details on AMF isolates). *Funneliformis mosseae* is relatively less cooperative than *R. irregularis* with respect to phosphorus exchange based on previous experiments manipulating plant and AMF combinations (see details on AMF species performance in Wagg *et al.* 2011; Argüello 2013). This design resulted in a full factorial combination of the six AMF treatments for each plant

142 species: 1) a non-AMF control whereby neither side of the root was inoculated (none:none);
143 2, 3) a non-AMF control on one side of the root system and a single AMF species on the other
144 side (none:AMF indicates either none:*F. mosseae* or none: *R. irregularis*); 4, 5) one AMF
145 species inoculated on both sides of the root system (same-AMF indicates either *F. mosseae*:*F.*
146 *mosseae* or *R. irregularis*:*R. irregularis*); 6) two AMF species with one applied to each side
147 of the root (two-AMF indicates *F. mosseae*:*R. irregularis*).

148 In order to standardize the bacterial communities among AMF treatments, all pots also
149 received 5 mL of a microbial wash. This wash was made by filtering (10 µm pore size) 5 L of
150 a suspension prepared from 25 g of the soil mixture and 25 g of each AMF inoculum. We
151 further applied 2.5 mL of rhizobium solution (OD_{580nm} of 0.2; *Rhizobium trifolii*, DSM 6040)
152 to ensure adequate nodulation of *Trifolium pratense*. All harvested roots of *T. pratense* had
153 active nodules.

154 *Growth conditions*

155 The microcosms were placed in a climate-controlled growth chamber with a 16/8 h
156 light/dark cycle, a temperature of 21/16°C (day/night), 60% relative humidity and an average
157 photosynthetic photon flux density of 400 µmol photons m^{-2} s^{-1}. The pots were watered every
158 other day with deionized water and their positions were randomly changed every week to
159 minimize differences in growth conditions for all the pots.

160 Plants were supplied with Hoagland solution (Hoagland & Arnon 1950) with half of
161 the normal P concentration (6 mM of KNO_3; 4 mM of $Ca(NO_3)_2$; 0,5 mM of NH_4NO_3; 1 mM
162 of $NH_4H_2PO_4$; 1 mM of $MgSO_4$; 50 µM of KCl; 25 µM of H_3BO_3; 2 µM of $MnSO_4$; 2 µM of
163 $ZnSO_4$; 0.5 µM of $CuSO_4$; 0.5µM of $(NH_4)6Mo7O_{24}$; 20 µM of Fe(Na)EDTA). Each root
164 compartment of the split-root system received 2 mL of this nutrient solution every second
165 week, just before the plants were watered, ensuring that the nutrients mixed well with the soil.

166 *^{14}C and ^{33}P labeling*

167 After 10 weeks, the polystyrene space holder was removed from the outermost
168 compartments and replaced by soil substrate containing 37 MBq of radio-phosphorus
169 ($H_3{}^{33}PO_4$). The other side received the same amount of non-radioactive phosphorus. Two
170 weeks after ^{33}P labeling, plants were pulse-labeled with $^{14}CO_2$ in a transparent acrylic
171 chamber. Throughout the labeling, the CO_2 concentration was monitored with an infrared gas
172 analyzer (LiCOR 6200, LiCOR, Nebraska) and maintained above 300 ppm by successively
173 releasing $^{14}CO_2$ from a sodium bicarbonate ($NaH^{14}CO_3$) solution by adding 5% H_2SO_4 with a
174 syringe. The air within the chamber was mixed with a fan. The chamber was maintained at a
175 reasonable temperature with a heat exchanger connected to an ice-water mixture.

176 The labelling was organized in sets of six microcosms (determined by the number of
177 microcosms that fit into the labeling chamber). These groups contained one pot with each
178 AMF combination for only one plant species. A total of 16 groups were processed
179 sequentially over a period of six weeks. Two consecutive groups formed a block, which were
180 identical in terms of plant and AMF species, but the radio-phosphorus label was applied to
181 opposite sides of the microcosms. For example, two pots with *P. lanceolata* inoculated with
182 *F. mosseae* on one side of the root system and *R. irregularis* on the other side were present in
183 two consecutive groups, but in the first, group ^{33}P was applied to *F. mosseae* while ^{33}P was
184 applied to *R. irregularis* in the second. Therefore, two pots with the same AMF treatment (e.g.
185 *F. mosseae*:*R. irregularis* and *R. irregularis*:*F. mosseae*) differ only in which side ^{33}P was
186 applied. This design was required in order to test the contribution of ^{33}P from a specific
187 fungus while accounting for the fungus on the opposite side of the root system and was
188 practically necessary because of limitations in the number of pots that could fit in the chamber
189 for ^{14}C labelling. In total, our experiment encompassed 96 microcosms (2 plant species × 6

AMF combinations × 2 pots of each combination in order to label opposite sides with ^{33}P × 4 replicates).

Destructive harvest

Harvesting of the microcosms began 112 hours after the ^{14}C-labelling was completed. Shoots were clipped, and roots were collected from both sides of the microcosms separately. Soil was recovered from all six compartments separately.

Fresh subsamples from shoots, roots on each side of the split-root system and soils from the root and hyphal compartments were ashed in a muffle oven (12 hours at 600ºC) and the residues dissolved in 2 mL 5.6 M HCl, followed by 5 mL H_2O. One milliliter of this solution was mixed with 4 mL Ultima Gold cocktail (Perkin Elmer, The Netherlands) and ^{33}P activity recorded by liquid scintillation counting (Tri-Carb 2900 TR, Packard, USA). Another 1 mL aliquot was used to determine total phosphorus concentration (San^{++} continuous flow analyzer, Skalar Analytical, The Netherlands).

A second subsample of the root and shoot material was dried (70 ºC, 72 h) and re-weighed. These samples were dry-combusted in a sample oxidizer (Model 307, Hewlett Packard, USA) involving trapping $^{14}CO_2$ in 10 mL Carbosorb (Perkin Elmer, The Netherlands) and addition of 10 mL Permafluor (Perkin Elmer, The Netherlands). ^{14}C activity was determined by liquid scintillation counting, and ^{14}C in soil samples was determined separately for the root and AMF compartments.

AMF colonization

Root subsamples were cleared with 10% KOH, followed by staining with 5% pen ink-vinegar mixture as described in Vierheilig *et al.* (1998). Stained roots were scored for the presence of AMF colonization using the intersect method outlined in McGonigle *et al.* (1990). For each sample, at 50 intersections of the root and a gridline the presence or absence of

hyphae, vesicles and arbuscules was recorded. From these measurements the total percentage of root length colonized by AMF (which equals the amount of root length occupied by hyphae) was estimated. All the sides of pots that were inoculated with AMF were colonized by AMF at the end of the experiment (Fig. 1c,d and Table S1). In some pots, there was root infection (although at lower levels) on the side that was not inoculated with AMF in the none:AMF treatment (Table S1). In all AMF treatments, infection was greater than 50%.

Statistical analyses

In order to assess resource transfer from the plant to the AMF, ^{14}C in the hyphal compartment (Bq) was analysed with a linear mixed-effects model as a function of the AMF treatment on the measured side (a fixed factor with 2 levels: *F. mosseae* and *R. irregularis*), the AMF treatment on the opposite side (a fixed factor with 3 levels: control, *F. mosseae* and *R. irregularis*) and a 2-way interaction between the two opposite root compartments. We used random effects for pot (a random term with 96 levels) and ^{14}C labelling group (a random term with 16 levels). A covariate for four consecutive ^{14}C labelling groups — which contained two ^{33}P labelling pairs for each species — was used to account for potential temporal variability in the ^{14}C labelling process that was inherent due to the inability to label all microcosms in a single chamber. A variable for plant species identity (a fixed factor with 2 levels; *P. lanceolata* and *T. pratense*) was also used as a main effect because no significant interactions between species and treatments were found. In order to meet the assumptions of homoscedasticity and to standardize for potentially unequal photosynthetic rates among plant species and replicates, ^{14}C was log transformed.

Total ^{33}P in the plant (Bq; above and belowground combined) was analysed with a linear mixed-effects model as a function of the AMF treatment on ^{31}P labelled side (a fixed factor with 3 levels; control, *F. mosseae* and *R. irregularis*), the AMF treatment on ^{33}P labelled side (a fixed factor with 2 levels; *F. mosseae* and *R. irregularis*; the control was

239 dropped as only negligible amounts of P were measured without AMF) and a 2-way
240 interaction between ^{31}P labelled side and ^{33}P labelled side. A covariate for four consecutive
241 ^{14}C labelling groups and a fixed term for plant species were also used (significant interactions
242 were not found between plant species and the AMF treatments for P labelling). We used a
243 random effect for labelling group (a random term with 16 levels). In order to meet the
244 assumptions of homoscedasticity and normalized residuals, ^{33}P was square-root transformed.

245 In order to assess resource exchange between the partners of the plant–AMF
246 mutualism, the ratio of ^{14}C in the hyphal compartment to ^{33}P in the plant was analysed with a
247 linear mixed-effects model as a function of AMF treatment on the ^{33}P labelled side (a fixed
248 factor with 2 levels; *F. mosseae* and *R. irregularis*), the AMF treatment on the ^{31}P labelled
249 side (a fixed factor with 3 levels; control, *F. mosseae* and *R. irregularis*) and their interaction.
250 The interaction is important to assess the effect of whether the treatment in the opposite
251 compartment altered resource exchange. A covariate for four consecutive ^{14}C labelling groups
252 and a fixed term for plant species were also used (significant interactions were not found
253 between plant species and the AMF treatments for the resource exchange ratio). We used
254 random effects for the labelling group (a random term with 16 levels). The ^{14}C to ^{33}P ratio was
255 log-transformed in order to meet the assumptions of homoscedasticity and normalized
256 residuals.

257 We analysed plant performance (total plant biomass in grams) as a function of plant
258 species, AMF treatment (a fixed factor with six levels; none:none, none:*R. irregularis*, none:
259 *F. mosseae*, *R. irregularis*:*R. irregularis, F. mosseae*:*F. mosseae* and two-AMF) and their
260 interaction. A covariate for four consecutive ^{14}C labelling groups — which contained two ^{33}P
261 labelling pairs for each species — was used to account for potential temporal variability in the
262 ^{14}C labelling process that was inherent due to the inability to label all microcosms in a single
263 chamber. We used random effects for labelling group (a random term with 16 levels). This

264 simpler model was used because plant biomass was independent of which side was labelled
265 with ^{33}P (e.g. in the two-AMF treatment *R. irregularis*:*F. mosseae* and *F. mosseae*: *R.*
266 *irregularis* are identical treatments). AMF performance (percent root colonization) was
267 analysed with the same models described for ^{33}P and ^{14}C in order to allow comparison.
268 However, all possible two-way interactions between plant species and treatments and a three-
269 way interaction among plant species, AMF treatment on one side and AMF treatment on the
270 other side were maintained in the model. Because of interactions between fixed terms, random
271 effects for treatment on the ^{33}P side nested in group (a random term with 48 levels) and
272 treatment on the ^{31}P side nested in group (a random term with 48 levels) were also used in the
273 model. All analyses were performed with the asreml-R package (ASReml 3, VSN
274 International, UK) in the R statistical software (version 3.1.2; http://r-project.org). Summary
275 of ANOVA results for all models are in Tables S1 and S2. All estimates in the results are
276 presented in the scale with which they were analysed (biomass = g, AMF infection = %, ^{14}C =
277 log Bq, ^{33}P = square-root Bq, and $^{14}C/^{33}P$ = log Bq Bq^{-1}).

278 **Results**

279 The total plant biomass of the two plant species was lowest with no AMF present on
280 the roots, but the two species responded differently to increasing AMF richness and increasing
281 number of inoculated root sides. *P. lanceolata* (the species less responsive to AMF)
282 responded similarly to both AMF species regardless if one root side or both were inoculated
283 (Fig. 2a), but *P. lanceolata* had significantly higher biomass with *F. mosseae* (the less
284 cooperative species) than with *R. irregularis* (the more cooperative species) when both sides
285 of the root were inoculated (Table S1a). Interestingly, *R. irregularis* had on average 13%
286 (95% CI: 8 – 18) greater infection with *P. lanceolata* roots than *F. mosseae* (Fig. 2c). *T.*
287 *pratense* (the species more responsive to AMF) had significantly lower biomass in the
288 none:AMF treatment with only one root side inoculated with *F. mosseae* than all other

289 treatments with inoculated roots (Fig. 2b, Table S1a). There was statistically no difference
290 between *F. mosseae* (66%, 95% CI: 61 – 71) and *R. irregularis* (65%, 95% CI: 60 – 70)
291 infection levels on *T. pratense* (Fig. 2d). The none:none AMF treatments did not contain any
292 AMF (Table S1).

293 Average allocation of ^{14}C to the hyphal compartment was significantly lower in *R.*
294 *irregularis* than in *F. mosseae* in treatments where only one species was present (i.e.
295 none:AMF and same-AMF; Fig. 3). However, when both species were present on opposite
296 sides of the root (two-AMF treatment) the difference between ^{14}C allocation was statistically
297 indistinguishable between the two AMF species (difference between allocation to *F. mosseae*
298 and *R. irregularis* in the two-AMF treatment = 0.3 log Bq, 95% CI: -0.1 – 0.7; Fig. 3). The
299 plant species responded similarly to the treatments and, on average, *P. lanceolata* contributed
300 significantly more ^{14}C to AMF than *T. pratense* (difference between ^{14}C in the hyphal
301 compartments between *P. lanceolata* and *T. pratense* = 0.5 log Bq, 95% CI: 0.3 – 0.7; Table
302 S3a). Small amounts of ^{14}C was measurable in the none:none treatment but were likely due to
303 root respiration and transmission through the soil by other soil microbes.

304 In none:AMF and same-AMF treatments, average ^{33}P in the plant was significantly
305 higher when provided by *R. irregularis* (e.g. difference between *F. mosseae*:*F. mosseae* and
306 *R. irregularis*:*R. irregularis* treatments = 18 square-root Bq, 95% CI: 12 – 24; Fig. 4).
307 However, in the presence of both AMF species on the roots, ^{33}P in the plant was higher from
308 *F. mosseae* (difference between ^{33}P contribution from *F. mosseae* and *R. irregularis* in the
309 two-AMF treatment = 11 square-root Bq, 95% CI: 3 – 20; Fig. 4), and this result was
310 consistent for both plant species (Table S3b). Furthermore, *F. mosseae* provided significantly
311 more ^{33}P to the plant in the presence of *R. irregularis* than in its absence (difference between
312 ^{33}P from *F. mosseae*:*F. mosseae* combination in the same-AMF and *F. mosseae*:*R. irregularis*
313 combination in the two-AMF treatment = 26 square-root Bq, 95% CI: 20 – 32). Plant ^{33}P was

not significantly different from zero in the none:none treatments (5 square-root Bq, 95% CI: -1 – 11). The results of plant ^{33}P were similar to that of total and relative plant phosphorus (Fig. S1,S2).

The cost of phosphorus in terms of carbon was significantly higher from *F. mosseae* than from *R. irregularis* when only one AMF species was present on the roots. The cost of phosphorus in terms of carbon from *F. mosseae* and *R. irregularis* in the same-AMF treatments was 350% higher (95% CI: 170 – 640; Fig. 5). However, the cost from the two AMF species became statistically indistinguishable when both AMF species were present (difference between cost of phosphorus from *F. mosseae* and *R. irregularis* in the two-AMF treatment = 0.2 log ^{14}C per ^{33}P, 95% CI: -0.4 – 1.7; Fig. 5). Although the cost of phosphorus from *R. irregularis* increased slightly — but not significantly — in the presence of *F. mosseae* (difference between cost of phosphorus from *R. irregularis* in the same-AMF treatment and *R. irregularis* in the two-AMF treatment = 0.3 log ^{14}C per ^{33}P, 95% CI: -0.2 – 0.9; Fig. 5), the cost from *F. mosseae* significantly decreased in the presence of *R. irregularis* (difference between cost of phosphorus from *F. mosseae* in the same-AMF treatment and *F. mosseae* in the two-AMF treatment = -1.2 log ^{14}C per ^{33}P, 95% CI: -1.6 to -0.8; Fig. 5). The plant species responded similarly to the treatments and, on average, the cost of phosphorus was higher for *P. lanceolata* than *T. pratense* (difference between ^{14}C per ^{33}P between *P. lanceolata* and *T. pratense* = 0.9, 95% CI: 0.6 – 1.3; Table S3c) due to the greater ^{14}C allocation to the hyphal compartment by *P. lanceolata* (Table S3a).

Discussion

In this study, we assessed plant carbon allocation and phosphorus acquisition in relation to two AMF species using microcosms with a split-root system and a partitioned rhizosphere in order to understand the mechanisms maintaining the plant–AMF symbiosis. We showed that the two AMF species provided phosphorus at significantly different carbon

prices when they grew on the plant in the absence of another fungal species. Interestingly however, the less cooperative fungus (i.e. the fungus that took more C per unit P delivered to the host) became more cooperative when another fungus was present, and the presence of the cooperative fungus (i.e. *R. irregularis*) decreased the overall carbon cost of phosphorus from this relatively less cooperative fungus (i.e. *F. mosseae*). These results indicate that the presence of multiple AMF (e.g. AMF diversity) can improve resource trade among AMF partners and, in the case of the more responsive plant species (i.e *T. pratense*), increase biomass. Thus, AMF diversity may support the maintenance of the plant–AMF mutualism by altering markets prices for resources, which may explain why plant roots in the field are usually colonized by a high diversity of AMF.

In the absence of alternative fungal options, *F. mosseae* — the less cooperative AMF — was able to obtain more ^{14}C in exchange for less ^{33}P than the more cooperative AMF, *R. irregularis*. Interestingly, this increased ^{14}C consumption with reduced ^{33}P exchange never decreased the host's biomass below that of the none:none treatments (i.e. the symbiotic association remained beneficial and did not reduce fitness; see Reynolds *et al.* 2005 for opposite example). Furthermore, it only significantly reduced biomass relative to the two-AMF treatments for the more fungal-responsive plant species (*T. pratense*; Fig. 2. Therefore, the plants either had an excess of resources that they were able to allocate to AMF due to improved fitness caused by the presence of AMF or were effective at implementing sanctions that inhibited the AMF from over-consuming ^{14}C (Kiers & Van Der Heijden 2006; Jones *et al.* 2015).

A surprising result is the improved cooperativeness of *F. mosseae* in response to the presence of *R. irregularis*, as demonstrated by the increased ^{33}P in the plant. This result supports the market concept of supply and demand altering resource value (Noë & Hammerstein 1994). The addition of a second AMF species likely increased the overall

supply of P and drove P prices down. Therefore, the cost in C per unit of P was reduced due to a more competitive market (Noë & Hammerstein 1994; de Mazancourt & Schwartz 2010). However, the addition of a second AMF species could also have altered sink strength for carbon and phosphorus and with that the resource exchange ratio. Another potential mechanism promoting cooperation is partner fidelity feedback whereby the presence of the cooperative fungus improves the vigor of the plant giving it a luxury of carbon and those benefits in turn lead to more resources to the fungi (Foster & Wenseleers 2006; Kiers & Van Der Heijden 2006). In support of this concept, both fungi and plants had slightly improved performance in the two-AMF treatment (i.e. more AMF infection and more plant biomass; Fig. 2). Interestingly, the improved performance was stronger for the more AMF responsive plant (*T. pratense*) and the less cooperative AMF species (*F. mosseae*). The response of the plant is not surprising as it should be expected that more AMF species have a greater benefit to a highly AMF responsive plant, but greater infection by the *F. mosseae* may also be due to the plant rewarding the improved cooperation of *F. mosseae*, or allowing greater infection due to its improved cooperation.

There are of course limitations to using a simplified experimental system. The partitioning of the rhizosphere may have reduced root-hyphae and hyphae-hyphae competition, which would alter spatial interactions and resource access (Verbruggen *et al.* 2012). This reduced competition may have altered hyphal growth through increased proliferation or extension. Furthermore, there is the possibility that compartments became infected with other AMF species, but this issue is a problem in many controlled AMF experiments because of contamination from endophytes and spores. However, colonization of root tips by invading AMF would need to compete with pre-established AMF hyphae and infection (Wagg *et al.* 2011). Although this experiment represents a simplified plant–AMF system in a homogenous environment, our results still provide important progress towards understanding the effect of fungal diversity on market prices and resource exchange.

In conclusion, our novel experimental manipulation of both resource exchange and the rhizosphere highlights the importance of multiple competing AMF in altering market prices which promotes cooperation in the plant–AMF mutualism. AMF options reduced the carbon cost per unit of phosphorus by mainly increasing the amount of phosphorus the AMF partner provided and not by reducing the amount of carbon provided by the plant. These results indicate the importance of AMF options for the persistence of the plant–AMF mutualism and allude to potential underlying mechanisms for the role of AMF diversity in promoting biodiversity effects.

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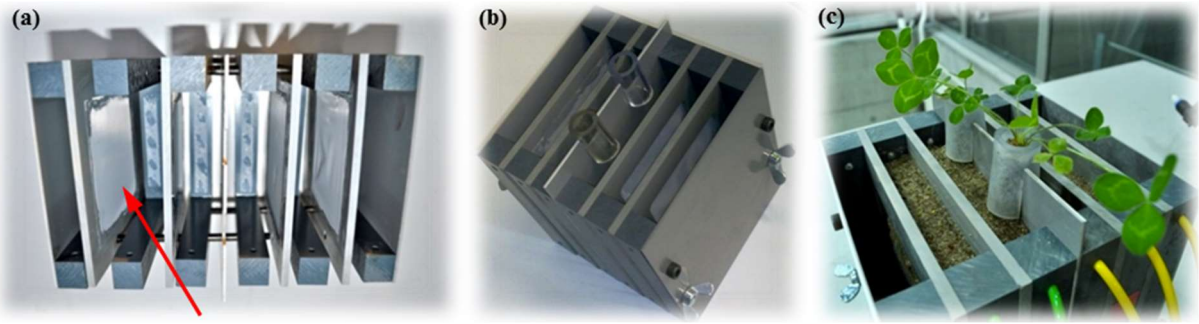


Fig 1. Schematic of split-root system and rhizosphere partitioning. (a) An aerial view of the partitions showing six compartments. The outer two were used for phosphorus addition. The middle two were hyphal compartments, and the inner two were root compartments housing the split-root system of the plant. The 20-μm mesh screen, which prevented root growth across partitions and ensured movement of nutrients via AMF, can be seen between the three compartments on each side (indicated by the red arrow). (b) A view of the entire system. (c) A view of the plants established with roots split onto either side of the main partition.

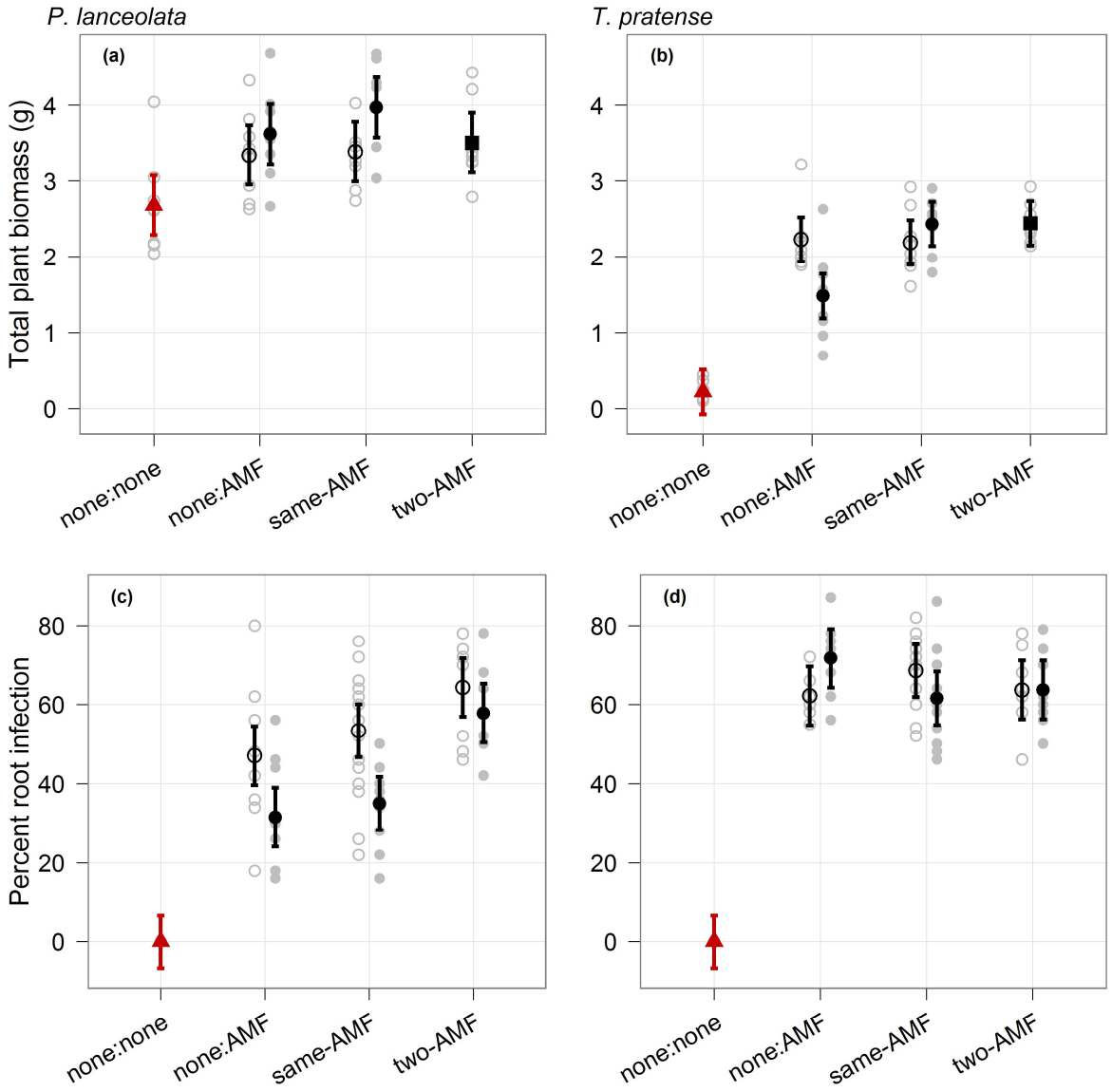
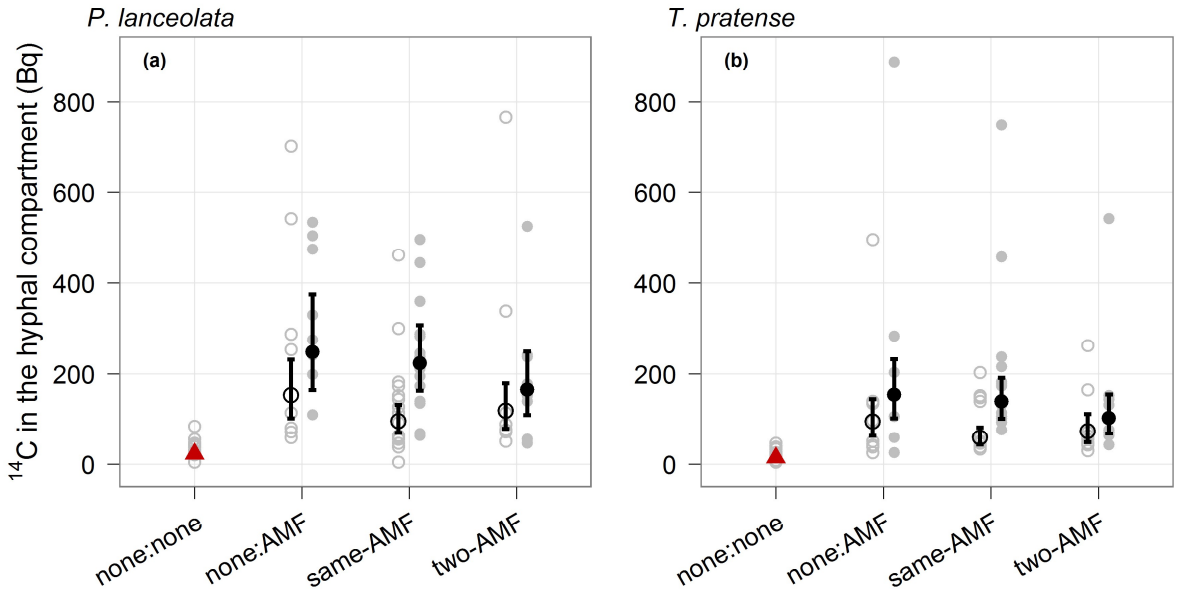


Fig 2. Total plant biomass and AMF root infection for each treatment. Mean biomass and infection (95% CI) is presented as a function of the AMF treatment on the ^{33}P labelled compartment and the interaction of the AMF treatment on the opposite side for (a, c) *P. lanceolata* and (b, d) *T. pratense*. The none:none treatments have no AMF, the none:AMF and same-AMF treatments have a single AMF species and the two-AMF treatments have a different AMF species on each side of the root. Colours represent the AMF species present in the treatment (black ○ = *R. irregularis*, black ● = *F. mosseae*, red ▲ = no AMF and black ■ = two-AMF). Large black points represent the model estimates, and grey points are observations.



536

537 **Fig 3. Total ^{14}C in hyphal compartment for each treatment.** Mean ^{14}C (95% CI) is
538 presented as a function of the AMF treatment in the hyphal compartment and the interaction
539 of the AMF treatment on the opposite side for (a) *P. lanceolata* and (b) *T. pratense*. The
540 none:none treatments have no AMF, the none:AMF and same-AMF treatments have a single
541 AMF species and the two-AMF treatments have a different AMF species on each side of the
542 root. Colours represent the AMF treatment on the side where ^{14}C was measured (black ○ = *R.*
543 *irregularis*, black ● = *F. mosseae* and red ▲ = no AMF). Large black points represent the
544 model estimates, and grey points are observations. The 95% CI does not encompass mean
545 estimates except in the two-AMF treatment. Data were back transformed from the natural-log
546 scale for presentation in the figure.

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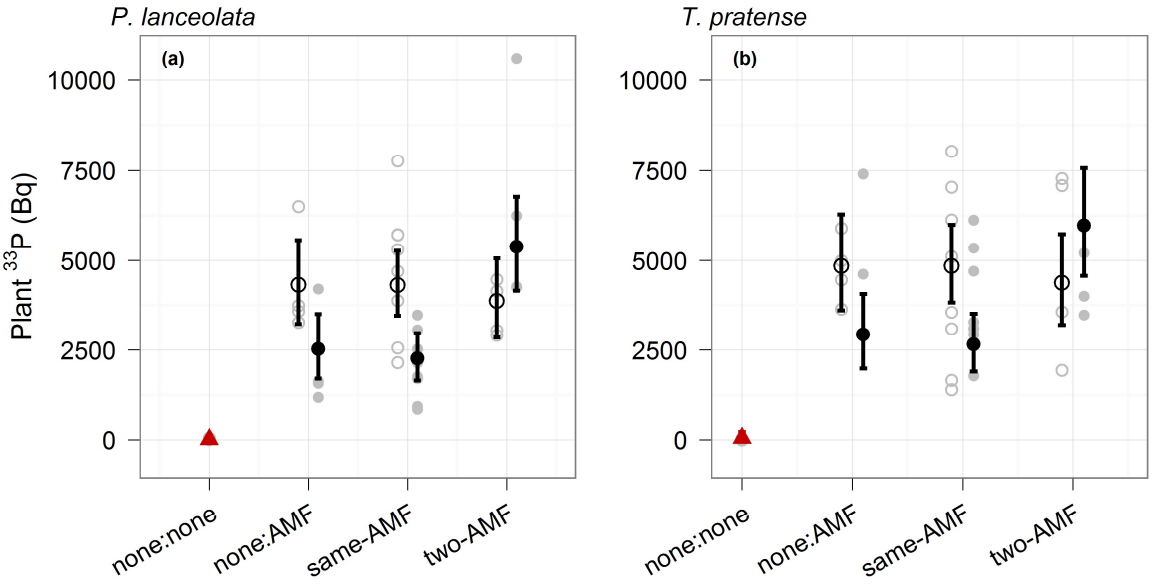
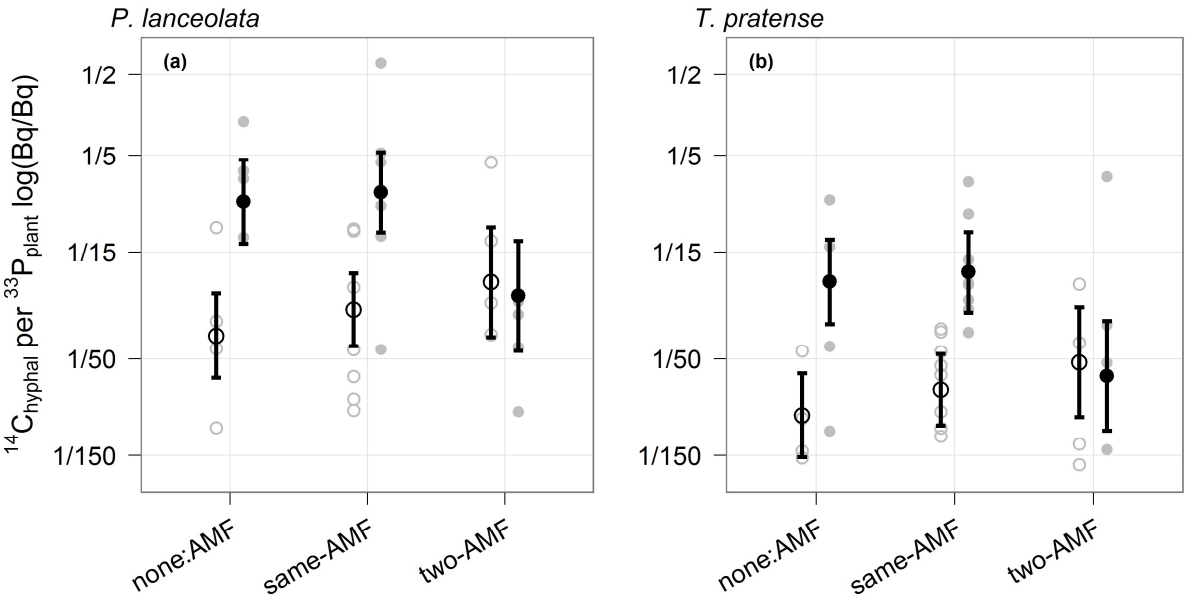


548

549 **Fig 4. ^{33}P in the plant for each treatment.** Plant ^{33}P (95% CI) is presented as a function of
550 the AMF treatment on the hyphal compartment and the interaction of the AMF treatment on
551 the opposite side for both (a) *P. lanceolata* and (b) *T. pratense*. The none:none treatments
552 have no AMF, the none:AMF and same-AMF treatments have a single AMF species and the
553 two-AMF treatments have a different AMF species on each side of the root. Colours represent
554 the AMF treatment on the side applied with ^{33}P (black ○ = *R. irregularis*, black ● = *F.*
555 *mosseae* and red ▲ = no AMF). Large black points represent the model estimates, and grey
556 points are observations. The 95% CI did not encompass mean estimates in any treatment. Data
557 were back transformed from the square-root scale for presentation in the figure.

558



559

560 **Fig 5. Resource exchange for each plant–AMF mutualism as a ratio of ^{14}C to ^{33}P.** The
561 cost carbon cost of phosphorus (95% CI) is presented as a function of the AMF treatment on
562 the hyphal compartment and the interaction of the AMF treatment on the opposite side for (a)
563 *P. lanceolata* and (b) *T. pratense*. The none:AMF and same-AMF treatments have a single
564 AMF species and the two-AMF treatments have a different AMF species on each side of the
565 root. Colours represent the AMF treatment on the side applied with ^{33}P (black ○ = *R.*
566 *irregularis* and black ● = *F. mosseae*). Large black points represent the model estimates, and
567 grey points are observations. The 95% CI does not encompass mean estimates except in the
568 two-AMF treatment.